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Alomar, Fadhel, Singh, Jaipaul ORCID: 0000-0002-3200-3949, Jang, Hee-Seong, Rozanzki, George J, Shao, Chun Hong, Padanilam, Babu J, Mayhan, William G and Bidasee, Keshore R (2016) Smooth Muscle-Generated Methylglyoxal Impairs Endothelial Cell-Mediated Vasodilatation of Cerebral Microvessels in Type 1 Diabetic Rats. British Journal of Pharmacology, 173 (23). pp. 3307-3326. ISSN 0007-1188

SMOOTH MUSCLE CELLS ARE THE SOURCE OF HEART FAILURE-CAUSING METHYLGLYOXAL IN DIABETES MELLITUS

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Diabetic cardiomyopathy (DC) is an established cause of morbidity and mortality in individuals with diabetes mellitus (DM). However, its molecular causes remain poorly defined. Supra-physiologic level of the α-oxoaldehyde methylglyoxal (MG) has emerged as a possible candidate since it can promotes many of the pathobiologies reported in DC. If and how MG production/flux is increased in the heart during DM remains unclear. Here ventricular tissues from patients and rats were used to assess protein levels of the MG synthesizing and degradation enzymes, vascular adhesion protein-1 (VAP-1) and glyoxalase-I (Glo-I), respectively. A rat model of DM was also used to determine if preventing Glo-I downregulation would be cardio-protective, establishing cause and effect. In ventricular tissues from patients and rats with DM, VAP-1 was upregulated in coronary microvascular smooth muscle cells (cSMCs) and Glo-I was reduced in cSMCs and myocytes. MG was also increased. Preventing Glo-I downregulation in cSMCs and myocytes of DM rats using a custom-engineered adeno-associated virus, lowered MG production/flux and blunted myocyte dysregulation and HF/DC. Preventing Glo-I downregulation blunted VAP-1 upregulation, prevented microvascular leakage, reduced oxidative stress and inflammation in ventricular myocytes, and minimized formation of MG adduct on and dysregulation of the sarcoplasmic reticulum Ca^{2+} cycling proteins, RyR2 and SERCA2a. These new data suggest that MG production/flux is enhanced in DM hearts via upregulation of VAP-1 in cSMCs. They also suggest that preventing Glo-I downregulation in cSMCs and myocytes could be a viable strategy to blunt diabetic DC development.

Number of words = 246

Nearly half a billion individuals worldwide have diabetes mellitus (DM), the syndrome that arises when insulin-producing β-cells of the pancreas are destroyed (Type 1 DM), the response of

peripheral organs/tissues to insulin is blunted (Type 2 DM), or both (usually late stage Type 2 DM) (1, 2). The good news is that diverse array of glucose-lowering agents, food management, exercise and education initiatives are available to help individuals with DM regulate their blood glucose levels to near physiologic levels (3-5). The not so good news is that a significant number of patients with DM are unable to regulate their blood glucose to the desired levels on a daily basis (4). One of the long-term consequences of inadequate glucose regulation is a 3-5 fold increase in the incidence of cardiovascular diseases (1). More recent data also suggest that in some patients with chronic Type 2 DM, tight glycemic control may promote cardiovascular morbidity and mortality (6-7), prompting a search for glucose-independent mechanisms that contribute to cardiovascular diseases in DM.

The heart is one of the organs whose function is negatively impacted by chronic/long-term DM. Longitudinal, cross-sectional and meta-analysis studies over the past forty years have confirmed this heart failure or diabetic cardiomyopathy (DC) as it is commonly referred to, starts as a delay in left ventricular relaxation (diastolic dysfunction) and gradually worsens to impairment of left ventricular contraction (systolic dysfunction) (8-11). Perturbation in myocyte intracellular Ca^{2+} handling, mitochondrial dysfunction, remodeling of the extracellular matrix/fibrosis, oxidative stress and inflammation, and endothelial dysfunction/microvascular disease, all contribute to the decline in left ventricular (myocyte) function (8-11). However, it is not clear whether these diverse pathobiologies seen in both Type 1 and Type 2 DM are originating from a single or multiple independent cues.

Methylglyoxal (MG) is an α-oxoaldehyde produced inside cells from the spontaneous and enzyme-catalyzed breakdown of the glycolytic pathway intermediates glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) (12-13). Thornalley estimated about

0.1% of glucotriose flux in red blood cells (RBCs) is converted to MG, equivalent to ~ 5.2 nmol MG/g RBCs/hour under normal glycemic conditions (14). Low MG regulates a diverse array of cellular and physiological functions including, cell differentiation, proliferation, anxiety, behavior, and sleep (15-22). Glyoxal is another structurally-related α-oxoaldehyde that it is generated from lipid peroxidation, the slow, spontaneous oxidative degradation of glucose and from degradation of glycated proteins. To date, its physiological role is not clearly defined (23), but it can be used as a marker of oxidative stress.

Several laboratories including ours have shown that supra-physiological amounts of α-oxoaldehydes, especially MG can elicit many of the pathobiologies reported in DC including impairment in endothelial cell function, perturbation in myocyte intracellular Ca^{2+} homeostasis, increases in ROS and inflammation and remodeling the extracellular matrix (24-29), making elevated MG a possible candidate for the varied pathobiologies seen in DC. However, is not clear if and how MG production/flux would increase in the heart during DM, especially since its primary source, glycolytic flux, is attenuated; the diabetic heart obtain >90% of the acetyl CoA needed for ATP production from β-oxidation of fatty acids (30). This dilemma led us to hypothesize that in the diabetic heart MG production/flux could increase if its production from non-glycolytic sources is increased or if its degradation is reduced.

Vascular adhesion protein-1 (VAP-1, *AOC3*, *EC 1*.4.3.21) is best known as the adhesin that is upregulated on high endothelial venules at inflammatory sites to aid in the extravasation of leukocytes from the blood into tissues (31). A less sialylated, but more glycosylated variant of VAP-1, is also expressed in low levels on the membrane of smooth muscle cells (SMCs) and adipocytes where it functions solely as the Cu^{2+} amine oxidase to deaminate the

glycine/threonine/tyrosine metabolite, aminoacetone to produce MG (32). VAP-1 is also upregulated by inflammation on smooth muscle cells.

MG (and glyoxal) is degraded in the cytoplasm of cells by the dual enzyme glyoxalase system (13, 33). Glyoxalase-I, (*GLOI*, EC4.4.1.5) is the rate-limiting enzyme and catalyzes the formation of *S*-2-hydroxyacylglutathione derivatives formed from the reaction of the α-oxoaldehydes and reduced glutathione (GSH). The second enzyme glyoxalase-II (*GLOII*, EC 3.1.2.6) which is also in the cytoplasm, catalyzes the hydrolysis of S-2-hydroxyacylglutathione derivatives to aldonates, liberating GSH. Three other cellular enzymes can also degrade MG (and other α-oxoaldehydes); aldehyde dehydrogenase, 2-oxoaldehyde dehydrogenase, and aldose reductase, albeit with significantly higher K_m values than Glo-I (34).

In this study we tested our hypothesis by first assessing steady state levels of VAP-1, Glo-I and MG levels in ventricular tissues from patients (Type 1 and Type 2), and in a rodent model of DM (streptozotocin-induced rats). We also used a gene transfer approach (an adeno-associated virus driven by the endothelin-1 promoter) to determine if preventing Glo-I downregulation in cSMCs and ventricular myocytes of DM rats would be sufficient to attenuate DC development later in the disease, establishing cause and effect.

Number of words = 797

METHODS

Antibodies and reagents

Primary antibodies use for the study were obtained from AbCam Inc, Cambridge MA (von Willerbrand factor, vWF, sheep polyclonal, Cat# ab11713, smooth muscle actin alpha, SM22α, rabbit polyclonal, Cat# ab14106, goat polyclonal, Cat# ab10135); Novus Biological LLC, Littleton, CO (mouse monoclonal SM22α, Cat# NBP2-00894); JaiCA, Shizuoka, Japan (argpyrimidine Arg, Cat# JAI-MMG-030N); Santa Cruz Biotechnology Inc., Santa Cruz, CA (Glo-I [FL-184], rabbit polyclonal, Cat# SC-67351; VAP-1 [E-19], goat polyclonal, Cat # sc-13741; and actin [1-19], goat polyclonal Cat# SC-1616), CalBiochem/EMD Bioscience, Billerica, MA (VAP-1 [TK8-14], mouse monoclonal, Cat# OP190); Invitrogen Life Technologies, Grand Island NY (phospholamban, Cat# MA3-922, phospho phospholamban Ser 16/17, Cat#702369, RyR2, Cat # PA5-36121, SERCA2a, Cat # MA3-919) and Cell Signaling Technologies, Danves MA (NF-κB p65 Cat #8242 and phosphor-p65 (Ser536, NF-κB), Cat#3031). Secondary antibodies were obtained from Invitrogen Life Technologies (goat anti-mouse IgG coupled to Alexa Fluor 594, Cat# A-11032, chicken-anti-rabbit IgG coupled to Alexa Fluor 488, Cat# A-21441, donkey anti-goat IgG coupled to Alexa Fluor 488 Cat# A-11055, donkey anti-goat IgG coupled to Alexa 594 Cat# A11058, donkey-anti-sheep IgG coupled to Alexa Fluor 594, Cat# A-11016); Santa Cruz Biotechnology, Inc. (Donkey anti rabbit IgG-HRP, Cat# sc-2305 Donkey anti goat IgG-HRP Cat# sc-2304) and Vector Laboratories, Burlingame, CA (Fluorescein rabbit anti-rat IgG Cat# FI-4000, and peroxidase-conjugated anti-mouse IgG).

Bovine serum albumin labeled with fluorescein isothiocyanate (BSA-FITC, Cat# A9771), 2,3,5-triphenyltetrazolium chloride (Cat# T8877), sodium thiobutabarbital (Inactin® Cat# T133)

and glyoxalase-I (Cat# G4252-500) were from Sigma-Aldrich (St Louis, MO). All other reagents used were of the highest grade commercially available.

Collection and preparation of autopsied ventricular tissues from DM patients

Rapid autopsied formalin-fixed, de-identified heart tissues from non-diabetic and diabetic patients (Type 1 and Type 2) were obtained from the Department of Pathology and Microbiology at the University of the Nebraska Medical Center (UNMC). Tissues were embedded into paraffin blocks by Tissue Core Facility at UNMC and 5 μm sections were cut and mounted on glass slides.

Immuno-histochemistry on paraffin-embedded human ventricular tissues

Immuno-histochemical staining was conducted on paraffin-embedded non-diabetic and DM sections for the SMC marker, smooth muscle actin (SM22α), VAP-1, Arg and Glo-I. For this, slides containing ventricular sections were heated to 65°C for 2 hr. Paraffin was then removed with xylene (3 changes, ten minutes each) and rehydrated in decreasing concentrations of ethanol (100%, 100%, 95%, 70%, 50%, and distilled water, three minutes each). Antigens were retrieved by incubating slides in 0.01 M citric acid (pH 6.39) for 40 min at 95 $^{\circ}$C. Sections were then cooled to room temperature for 20 min and then blocked in 10% normal horse serum for 1 hour at RT and incubated overnight at 4 $^{\circ}$C with the respective primary antibodies (1:100 to 1:200). The next day, sections were incubated with the corresponding secondary Alexa Fluor coupled antibodies (1:400 to 1:800). Sections were then washed with PBS and mounted with Prolong Gold Mountant Anti-fade containing DAPI. Images were then taken with a Nikon inverted fluorescence microscope (TE 2000) equipped with a CoolSNAP HQ2 CCD Camera (Photometrics, Tucson, AZ). A Nuance EX multispectral imaging system (Cambridge Research

Instruments, Worburn, MA) fixed to a Nikon Eclipse E800 and image analysis software (Caliper Life sciences, Inc., a Perkin Elmer Company, Hopkinton, MA) was used to quantitate changes of VAP-1, MG and Glo-I immunoreactivities (36) in a blinded manner using three consecutive 100 X frames.

Construction of adeno-associated viruses

The University of Pennsylvania Vector Core Facility generated the adeno-associated viruses containing rat glyoxalase-I used in this study with support from the Gene Therapy Resource Program, GTRP # 1053. More details on construction of rat Glo-I into the plasmid are provided in our recent publication (35).

Induction, verification and treatment of DM rats

Animal used for this study were approved by the Institutional Animal Care and Use Committee, University of Nebraska Medical Center and adhered to the Guide for the Care and Use of Laboratory Animals. DM was induced in anesthetized male Sprague-Dawley rats using a single low-dose intravenous injection of streptozotocin (STZ, 40-45 mg/kg in 0.1 mL in citrate buffer pH 4.5). Control animals were injected with the same volume of citrate buffer only as detailed earlier (29, 36).

One week after injection of STZ (blood glucose >300 mg/dL), DM rats were randomly divided into four groups. The first group received a single intravenous injection of AAV2/9-Glo-I driven by the endothelin-1 promoter (1.7×10^{12} viron particles/kg in sterile physiologic saline solution, *DM-Endo-Glo-I*), the second group received a single injection of AAV2/9-Endo-eGFP (a non-specific gene, 1.7×10^{12} viron particles/kg, *DM-Endo-eGFP*), the third remained untreated

for the duration of the 8 week study (*DM*) and a fourth group was injected with AAV2/9-Glo-I driven by the more global cytomegalovirus promoter (1.7×10^{12} viron particles/kg, *DM-CMV-Glo-I*). Rats injected with citrate buffer only (*Con*) were divided into two groups. The first control group was injected with AAV2/9-Endo-eGFP (*Con-Endo-eGFP*), and the second group remained untreated (*Con*) for the duration of the study. The endothelin-1 promoter was selected to drive expression of Glo-I in AAV2/9 since prior studies have shown that SMCs and myocytes express endothelin-1 (35). The CMV promoter was used since it should increase expression of Glo-I expression in all cell types of the heart infected (37). The dose (multiplicity of infection) of virus used was selected from our recent study (35) and is in agreement with other studies (38-39).

Well-being of animals

Nest construction was used as a measure of the well-being of animals (35, 40). Two hours prior to start of a dark-cycle, rats were transferred to clean, dry cage with a piece of cotton (11 ± 0.5 g, PetCo San Diego, CA) placed in one corner. The next day, a five-point scale was used to score overnight nest construction; shredding and movement of <10% of cotton to center of cage = 1; shredding and movement of ~25% cotton to center of cage = 2; shredding and movement of ~50% to the center of cage = 3; shredding and movement of ~75% of cotton to the center of cage = 4; shredding and movement of 100% to the middle of cage = 5.

Ventricular function in rats

At the end of the 8 week study protocol, hair from the chests of rats was removed (Nair, Church & Dwight Co., Inc. NJ, USA). The next day, rats were anesthetized with 5% isoflurane, taped in the supine position on a heated (37°C) pad and Pulse-wave echocardiography was performed to

assess peak flow velocity during early diastole (E) and peak flow velocity during atrial contraction (A) using a color flow–guided, pulsed-wave Doppler probe (716, 17.6-MHz). M-mode echocardiography (short axis orientation) was also conducted to assess ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD), percent fractional shortening (% FS), percent ejection fraction (% EF), stroke volume and cardiac output using a built-in algorithm (29, 36). All echocardiographic studies were performed using a Vevo 770 High Resolution in Vivo Micro Imaging System **(**Fuji Film Visual Sonics Inc, Toronto Canada).

Microvessel perfusion and permeability

At the end of the 8-week protocol rats were injected intraperitoneally with heparin (100 unit/kg, i.p.). Five min later, rats were anaesthetized (100 mg/kg sodium thiobutabital i.p.) and a single injection of bovine serum albumin coupled to fluoroscein isothiocynate (BSA-FITC, 40 mg/kg in sterile 1X PBS buffer, 50 µL) was injected via a vein under the tongue and allowed to circulate for 10 min (35). After this, animals were euthanized, chest cavities were opened and hearts were quickly removed and immersed in 4% paraformaldehyde for 24 hr at 4°C. The next day, hearts were transferred to 4% paraformaldehyde/15% sucrose solution for 24 hrs, followed by 4% paraformaldehyde/30% sucrose solution for 24 hrs, and then 30% sucrose solution for 24 hrs before storing at -80 °C.

Cryoprotected rat hearts were then cut into 20 µm thick longitudinally sections on a microtome (Leica EM-UC 6, Leica Microsystems, Wien, Austria) and mounted onto pre-cleaned glass slides. Slices were then washed three times with 1X PBS to remove cutting medium. Vectashield mounting medium containing DAPI was added to the sections, slides were cover slipped and placed on the headstage of a Nikon TE2000 microscope attached to a Coolsnap HQ2

CCD camera (Photometrics, Tuscon AZ). Images were then taken to assess the density of microvessels perfused with BSA-FITC, and for leakage of microvessels. For determining the density of perfused microvessels, 100 X frames from three adjacent sections were analyzed. To be counted as a perfused vessel, the vessel must contain BSA-FITC (green) in a length $\geq$ 40 μm. Branched vessels were counted as one vessel. A vessel was counted as leaky if BSA-FITC was seen emanating from the confines of its microvessel walls.

Isolation of rat ventricular myocytes

Ventricular myocytes were isolated using the procedure described earlier (29, 36). For this, rats were injected intraperitoneally with heparin (1000 U/kg) 10 min prior to sacrifice with a lethal dose of Inactin® (75 mg/kg, i.p.). Chest cavities were opened, and hearts were removed with the aorta intact. Excised hearts were placed in a Krebs–Henseleit (K–H) buffer (13.8 mM NaCl, 4.0 mM KCl, 1.2 mM $MgSO_4$, 0.9 mM NaH_2PO_4, 25 mM $NaHCO_3$, 8.4 mM HEPES, 11.1 mM D-glucose, 1 mM adenosine) and adventitious tissue around the aortas were removed. Hearts were then mounted in the Langendorf configuration and perfused for 5–6 min at a flow rate of 6–8 mL/min with K–H buffer containing 1.8 mM $CaCl_2$ to remove blood from the vessels. The perfusion solution was then switched to Ca^{2+}-free K–H and the hearts were perfused for an additional 10 min. Hearts were then perfused in a recirculatory manner with Ca^{2+}-free K–H buffer containing Type 2 collagenase (0.66–0.80 mg/mL) for 25–28 min. After digestion of the extracellular matrix, hearts were removed from the perfusion apparatus and the atria and aorta were trimmed away. Ventricular tissues were then finely cut in buffer containing 0.25 mM Ca^{2+} and trituated to enhance myocyte release. Cells were filtered through nylon meshes (300 μm) and collected into 14 mL polypropylene cell culture tubes. Cells were then incubated for 10 min

at 37 ºC with gentle rotation. Cells were then allowed to settle and half the supernatant was removed and replaced with K–H buffer containing 0.25 mM Ca^{2+}. The process was repeated with 0.5 mM, 0.75 mM and 1.0 mM Ca^{2+} to gradually restore Ca^{2+} tolerance. Cells were resuspended in Dubelcco' modified eagle medium containing 1.8 mM Ca^{2+}, supplemented with F-12 (1:1) and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin and 100 μg/mL gentamicin), pH 7.3, placed in a water-jacketed incubator at 37 °C and used within 5–6 hr after isolation.

Contractile kinetics of rat myocytes

A cohort of ventricular myocytes from each group of animals was used for assessment of mechanical properties using a high-speed video-based edge detection system (IonOptix Corporation, Milton, MA) (29, 36). For this, myocytes in DMEM were placed in a chamber mounted on the stage of an inverted microscope (Zeiss X-40, Thornwood, NJ) at room temperature (22 ºC – 24 ºC). Cells were then field stimulated (10 V, 10 ms at 0.5 Hz) using a pair of platinum wires placed on opposite sides of the chamber and contraction/relaxation were recorded using a proprietary data acquisition program (IonOptix Corporation, Milton, MA). Rates of myocyte shortening, relengthening, and extent of cell shortening were calculated using IonWizard, Version 5.0.

Evoked SR Ca^{2+} release in rat ventricular myocytes

A cohort of ventricular myocytes from each group was used to assess evoked Ca^{2+} release (29, 36). For this, freshly isolated ventricular myocytes attached to laminin-coated coverslips in DMEM-F12 medium were loaded with Fluo-3 (5 μM) for 30 min at 37 ºC. After this, cells were washed to remove extracellular Fluo-3 and placed in a chamber on the stage of the confocal

microscope (Zeiss 510 confocal microscope, Carl Zeiss Inc., Thornwood, NY). Cells were then field stimulated (0.5 Hz, 10 V, 10 ms) using a pair of platinum wires, and changes in fluorescence intensities (ΔF) were determined. Fluo-3 was excited by light at 488 nm, and fluorescence was measured at wavelengths of >515 nm. All experiments were performed in line-scan mode. LSM Meta 5.0, Prism 5.0, and Microsoft Excel were used for analyzing rates of Ca^{2+} rise (linear regression) and decay constants (one-phase exponential decay).

Immuno-histochemistry on paraformaldehyde-fixed rat ventricular tissues

Immuno-histochemistry was also conducted on rat ventricular slices for VAP-1, argpyrimidine, Glo-I, and the smooth muscle actin alpha (SM22-α). For this, 20 μm ventricular slices were permeabilized in 1X phosphate buffered saline containing using 0.1% Triton-X 100 and 10% normal horse serum for 1 hr at room temperature. Slices were then washed three times with 1X PBS (5 min) and incubated with primary antibodies (1:100 to 1:200) for 16 hrs at 4 °C. At the end of the incubation, heart sections were washed three times with 1X PBS, and incubated in the dark for 1 hr with appropriate secondary antibodies coupled to Alexa Fluor 488 and 594 antibodies (1:400 to 1:600) at room temperature. Slices were then washed three times with 1 X PBS. Vectashield mounting medium with DAPI was then added and slides were cover slipped. Images were then collected on a Nikon TE 2000 microscope and the Nikon Eclipse E800 and image analysis software (Caliper Life sciences, Inc., a Perkin Elmer Company, Hopkinton, MA) was used for quantitate changes of VAP-1 and Glo-I.

Steady-state levels of sarco(endo)plasmic reticulum Ca^{2+} cycling proteins, VAP-1 and Glo-I

Western blot assays were used to determine steady state levels of type 2 ryanodine receptor (RyR2), sarco(endo)plasmic reticulum Ca^{2+}ATPase (SERCA2a), phospholamban (monomeric and pentameric), argpyrimidine (a MG adduct) VAP-1 and Glo-I using procedures described earlier (29, 35, 36). For this, ventricular tissues (200 mg) were cut into small pieces and placed in 600 μl of ice-cold buffer (0.3 M sucrose, 10 mM histidine and 230 μM of freshly dissolved phenylethylsufonyl fluoride, in 100% ethanol, pH 7.4) for 10 min. Tissues were homogenized for 3 X 5 sec (15 sec intervals homogenization, ProScientific, PRO 25, setting 4.5, Oxford, CT). Samples were then centrifuged for 10 min at 3000 rpm. The supernatants were removed and protein concentrations were determined. Protein samples (60-80 μg) were then dissolved in gel dissociation medium (62.5 mM Tris base, 6% SDS, 20% glycerol, and 0.002% bromphenol blue containing 12 mg dithiothreitol/mL) and heated to 50-55ºC for 15 minutes. After this, samples were electrophoresed using 4–15 or 4 to 20% linear gradient polyacrylamide gels at 150 V for times varying from 1.0 to 3.5 hr, depending on protein of interest. After electrophoresis, proteins were transferred onto polyvinyldiethylene difluoride membranes, blocked (0.01 M Tris-HCl, 0.05 M NaCl, 5% bovine serum albumin, Sigma , and 0.04% Tween 20, pH 7.4) for 1 hr and incubated overnight with respective primary antibodies (1:1000), followed by the secondary antibodies (1:2000-2500) coupled to horse radish peroxidase. Membranes, were exposed to a 1:1 dilution of ECL™ (or enhanced ECL™) Western Blot Detection Reagents (Invitrogen, NY) for 2 min and then exposed to blue ultra autoradiographic film 8x10, double emulsion blue (ISC BioExpress, Kaysville, UT). β-actin served as the internal control to correct for variations in sample loading.

Immunohistochemical assays were also performed to determine locations of VAP-1 and Glo-I in rat ventricular tissues rats. For this, 20 μm ventricular slices were permeabilized in 1X phosphate buffered saline containing using 0.1% Triton-X 100 and 10% normal horse serum for 1

hr at room temperature. Slices were then washed three times with 1X PBS (5 min) and incubated with primary antibodies (1:100 to 1:200) for 16 hrs at 4 °C. At the end of the incubation, heart sections were washed three times with 1X PBS, and incubated in the dark for 1 hr with appropriate secondary antibodies coupled to Alexa Fluor 488 and 594 antibodies (1:400 to 1:600) at room temperature. Slices were then washed three times with 1X PBS. Vectashield mounting medium with DAPI was then added and slides were cover slipped. Images were then collected on a Nikon TE 2000 microscope and the Nikon Eclipse E800 and image analysis software (Caliper Life sciences, Inc., a Perkin Elmer Company, Hopkinton, MA). Smooth muscle actin alpha (SM22-α) was used as a marker for smooth muscle cells of microvessels.

Activities of sarco(endo)plasmic reticulum Ca^{2+}cycling proteins, VAP-1 and Glo-I

(a) SERCA2a activity: Ca^{2+} uptake assays (steps 1–6 of the post-Elbers cycle [$E_1 \rightarrow E_2$]) were also used to assess the activity of SERCA2a (36). For this, sarcoplasmic reticular membrane vesicles were resuspended in 1 mL buffer (30 mmol/L Tris-HCl, pH 7.0; 100 mmol/L KCl; 5 mmol/L NaN_3; 5 mmol/L $MgCl_2$; 0.15 mmol/L EGTA; 0.12 mmol/L $CaCl_2$; 1 μCi $^{45}Ca^{2+}$; and 10 mmol/L potassium oxalate) and were divided into 2 × 500 μL aliquots. One aliquot was incubated with a thapsigargin (10 μmol/L), ammonium molybdate (100 μmol/L), bafilomycin (0.05 μg/mL) and ryanodine (50 μmol/L) for 15 min and the other remained untreated. Ca^{2+} uptake was initiated by adding Na_2ATP (3 mM) and aliquots (50 μL) were taken at 0, 10 and 20 min and filtered using Whatman GF/C filters (1.2 μm). The filters were then rinsed twice by using 3-mL portions of ice-cold buffer (without $^{45}Ca^{2+}$). A 50 μL aliquot was taken from each tube to verify the total $^{45}Ca^{2+}$ content. Intravesicular $^{45}Ca^{2+}$ was determined by liquid scintillation counting of the protein retained on the filters.

Ca^{2+}-ATPase activity (steps 1–3 of the post-Elbers cycle) of SERCA2a was also assessed using the procedure earlier (36). Briefly, membrane vesicles containing SERCA2a (equivalent amounts of proteins) were incubated in 500 μL buffer (10 mmol/L HEPES, pH 7.3; 0.1 mol/L KCl; 5 mmol/L Mg^{2+}; 100 μmol/L Ca^{2+}; 100 μmol/L EGTA; and 2.5 mmol/L Na_2-ATP) and incubated for 20 min at 37°C in the presence and absence of the Ca^{2+} ionophore A23187 (2 μg/mL). After this time, reactions were stopped and the inorganic phosphate generated from ATP hydrolysis was assessed using the malachite green colorimetric assay described previously.

(b) RyR2 activity: Ca^{2+}-dependent binding of [^{3}H]ryanodine (29) was used as an index of RyR2 function. In this assay, [Ca^{2+}] ≤300 μM activate or open RyR2 thereby increasing [^{3}H]ryanodine binding, while higher Ca^{2+} concentrations deactivate or close RyR2, decreasing [^{3}H]ryanodine binding. For this, sarcoplasmic reticular membrane vesicles (0.1 mg/mL) prepared as described earlier (29) were incubated in binding buffer (500 mM KCl, 20 mM Tris–HCl, 5 mM reduced glutathione, 100 μM EGTA, pH 7.4) for 2 hrs at 37 °C with 6.7 nM [^{3}H]ryanodine and increasing amounts Ca^{2+} (13 nM to 5 mM). After incubation, proteins were filtered and washed, and the amount of [^{3}H]ryanodine bound to RyR2 was determined using liquid scintillation counting. Non-specific binding was determined simultaneously by incubating vesicles with 1000 nM unlabeled ryanodine.

(c) Glyoxlase-I activities in rat ventricular homogenates were determined spectrophotometrically using procedure described earlier (35). Briefly, in a 1 mL quartz cuvette, 250 μL of 0.1 M $NaPO_4$ buffer, pH 6.6, 233 μL of distilled water, 25 μL of 4 mM MG, and 50 μL of 2 mM reduced GSH were mixed and the absorbance at 240 nm was measured. Thereafter, 500 μg of homogenate from each group of animals were mixed and absorbance was recorded after 60 and 120 sec at room temperature using a spectrometer (Smart Spec 3000, BioRad Inc, Burlingame

CA). A standard curve was generated using commercially available Glo-I standard (1.63 units per ug) and used for quantification.

(d) VAP-1 activities in rat ventricular homogenates were assessed using SSAO assay kits (Cell Technology, Inc, Mountain View, CA) as per manufacturer instructions without modification.

Determination of methylglyoxal and glyoxal levels in rat serum and ventricular tissues

Methylglyoxal and glyoxal concentrations in serum and ventricular tissues were determined using a method optimized in our laboratory based on methods previously described by McLellan *et al.* (41-43). Briefly, serum (500 µL) and rat ventricular tissues (~200 mg, finely chopped) were placed in 3X volume or 3X weight/volume of cold isolation buffer containing 0.3 M sucrose, 10 mM histidine and 230 µM of freshly dissolved phenylethylsufonyl fluoride in 100% ethanol, pH 7.4. Samples were placed on ice for 10-15 min. After this, samples were homogenized for 3 X 5 sec (15 sec interval wait in between homogenization, ProScientific, PRO 25, setting 4.5). The homogenized samples were mixed thoroughly with equal volumes of perchloric acid (5.0 M) and placed on ice for 10 min. The samples were then centrifuged for 10 min at 3000 rpm (Eppendorf 5417R, EL 129 FA 45-24-11 rotor). Equal volumes of 0.1 M o-phenylenediamine prepared in 1.6 M perchloric acid were then added to each sample, mixed, wrapped around in aluminum foil and allowed to react overnight at room temperature. The reaction products were then extracted with 3 X 3 mL of chloroform and water was removed using anhydrous sodium sulfate (0.5 g). The chloroform from each extract was removed using a gentle stream of nitrogen. The dried samples were then resuspended in 500 µL methanol and stored at -80°C until analyzed. High performance liquid chromatography was performed of 5 µL injection of each sample in duplicate using (SCL-Shimadzu 10A, HPLC) employing a Kinetex 5µ C18 100R column using mobile

phase consisting of methanol: water: trifluoroacetic acid (52:48:0.1) at flow rate of 1 mL/min and wavelength of 312 nm. Calibration curves were generated using 5 µL injections of varying concentrations (0.5 to 50 µM) of 2-methylquinoxaline (cat #W511609, Sigma-Aldrich, St Louis MO) and quinoxaline (cat # Q1603 Sigma-Aldrich). In some samples (control and DM), o-phenylenediamine was left of from the reaction mixture and in some of the prepared samples, 50 µM mixture of 2-methylquinoxaline and quinoxaline were spiked in to identified peaks the retention times of the peaks of interest.

Glutathione (GSH) and enzymes that regulate GSH production

Glutathione (GSH) was measured in ventricular homogenates using a commercial kit according to manufacturer's protocol (Oxis Research, Portland, OR). Briefly, finely chopped ventricular tissues (~100 mg) from the various groups of animals were sonicated in 5% metaphosphoric acid and centrifuged at 4°C for 20 min (3000 X *g*) to precipitate proteins. Supernatants were collected and total glutathione [GSH + oxidized glutathione (GSSG)] was determined by measuring the formation of 2-nitro-5-thiobenzoic acid at 412 nm (25°C) in the presence of 5,5′-dithio-bis-(2-nitrobenzoic acid), NADPH, glutathione reductase, and EDTA. GSSG was also determined by derivatizing supernatants with 1-methyl-2-vinylpyridium trifluoromethane sulfonate and assaying the derivatized samples as above for total GSH. Standard curves for GSH and GSSG were constructed, and the [GSH] was calculated by subtracting the GSSG concentration from total glutathione (GSH+GSSG). Measured concentrations of GSH and GSSG were expressed in micromoles per liter per milligram protein and as a ratio (GSH/GSSG).

Glutathione reductase and γ-glutamylcysteine ligase were also measured in ventricular tissues (35, 44). Briefly, ventricular tissues (~200 mg) were homogenized in 600 µL of ice-cold Tris

buffer (0.1 mol/L, pH 8.0, with 2 mM EDTA) and centrifuged at 4°C (6,000 *g*) for 30 min as described earlier. A 200 μL aliquot of the supernatant from each group of animals was added to a cuvette containing KH_2PO_4 buffer (0.2 M, pH 7.0) plus 2 mM EDTA, 20 mM GSSG, and 2 mmol/L NADPH. Changes in absorbance at 340 nm were monitored over 5 min at 25°C using a spectrophotometer. A milliunit (mU) of glutathione reductase activity was defined as the amount of enzyme catalyzing the reduction of 1 nmol NADPH per minute.

γ-Glutamylcysteine ligase activity was also determined (35, 45). Ventricular homogenates were prepared as above and a 50-μl aliquot of the supernatant from each of animals was added to a reaction mixture containing 0.1 M Tris buffer and (in mM) 150 KCl, 5 Na_2ATP, 2 phosphoenolpyruvate, 10 L-glutamate, 10 L-α-aminobutyrate, 20 $MgCl_2$, 2 Na_2-EDTA, 0.2 NADH, 17 μg pyruvate kinase and 17 μg lactate dehydrogenase. Changes in absorbance at 340 nm were monitored for 5 min at 25°C, and γ-glutamylcysteine ligase activity was expressed in milliunits, defined as the activity converting 1 nmol of NADH to NAD per minute.

Measurement of oxidative stress and inflammation

Increases in oxidative stress and inflammation are characteristic features in the heart during DM (46-47). Earlier we showed that acutely exposing ventricular myocytes from control rats to MG increased mitochondria production of reactive oxygen species (ROS) (29). We also found increased production of the pro-inflammatory mediator, tumor necrosis factor alpha (TNF-α) at sites of vascular leakage (35). These findings prompted us to investigate whether increasing Glo-I in SMCs and myocytes of DM rats would lower ROS and inflammation.

(a) Measurement of ROS: Freshly isolated ventricular myocytes were loaded with MitoTracker Green (100 nM, Life Technologies, Grand Island NY), followed by the fluorogenic,

mitochondria-targeted, reactive oxygen species (ROS) probe MitoSOX™ Red (2 μM), for 15 min each. Cells were then washed 3X with fresh medium and placed on the stage of a Zeiss LSM 510 Meta laser scanning microscope, The red fluorescence images of MitoSOX™ Red was captured by exciting at 488 nm with emission at 594 nm, while the green fluorescence of MitoTracker Green was collected by excited at 488 nm, with emission at 516 nm (29, 36). MitoSOX™ Red fluorescence was quantified with ImageJ analysis software (http://rsbweb.nih.gov/ij/).

(b) Measurement of NF-κB activity: Activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) results in increased expression of pro-inflammatory genes including VAP-1 (48). In this study activation of this pathway was assessed by measuring the amount of phosphorylation of the p65 subunit of NF-κB using western blot assays. For this, left ventricular tissues (100 mg) were cut into small pieces and placed in 300 μL of ice-cold lysis buffer (300 mM NaCl, 40 mM Tris-HCl, 2 mM EGTA, and 2 mM EDTA, 1% Triton X 100, 0.1% SDS, phosphatase inhibitor cocktails 1 and 2, pH 7.4) for 10 min. Tissues were then sonicated for 3 X 5 sec (15 sec intervals between sonications). Lysates were then centrifuged for 10 min at 3000 rpm. Protein concentrations of the supernatants then determined. Lysates (75 μg) were then electrophoresed using 4–15% linear gradient polyacrylamide gels at 150 V for 1.5 hr. After this, proteins were transferred onto polyvinyldiethylene difluoride membranes, blocked with 5% non-fat milk for 1 hr and incubated overnight with phosphor-p65 (Ser536) and p65 antibodies (1:1000), followed by the secondary antibodies (1:2000-2500) coupled to horseradish peroxidase. Thereafter, membranes, were exposed to a 1:1 dilution of ECL™ Western Blot Reagents (Invitrogen NY) for 2 min, exposed to autorad film (ISC BioExpress, Kaysville, UT) and the intensities of the signals were used as indices of the amount of protein. Actin served as the internal control to correct for variations in sample loading.

(c) Measurement of TNF-α in rat ventricular tissues: Total RNA was extracted from freshly isolated, finely chopped rat ventricular tissues using Trizol and purified using RNeasy Mini Kit according to manufacturer's protocol (Qiagen; Germantown, MD) described earlier (35). RNA samples were treated with DNase I to remove any contamination DNA. RNA concentrations and purity were measured at OD 260 and OD 280. Total RNA with OD_{260}/OD_{280} greater than 1.8 (1 μg) were reverse-transcribed into cDNA using MLV reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA) in 20 μL volume. cDNA of each sample was used in Taqman real-time PCR to obtain the CT value for TNF-α in an ABI sequence detector (Applied Biosystems, Foster City, CA, USA). The real-time PCR for β-actin was run in parallel and served as the PCR control. Each sample was run in triplicate in PCR to determine gene expression of TNF-α. Primers used for TNF-α were *5'* ATG-AGC-ACT-GAA-AGC-ATG-AT and 3' CTC-TTG-ATG-GCA-GAG-AGG-AG (Integrated DNA Technologies (Coralville, IA). Relative gene expression (RGE) was calculated from the CT values using the Delta CT method.

Statistical Analysis

Differences among each of the groups were evaluated using one-way analysis of variance (ANOVA) followed by the Bonferroni's post-hoc test to determine if there was significant difference between Con and DM groups, and whether these changes were attenuated with treatment by using Prism GraphPad 6 (La Jolla, CA). Data shown are means ± S.E.M. Results were considered significantly different if $p<0.05$ (95% confidence interval).

Number of words 4653

RESULTS

The general characteristics of non-diabetic and patients from which autopsied tissue samples were obtained are shown in Table 1. There were no significant differences in mean age of non-diabetic and DM patients and time to harvest tissue after death. All patients had carried a diagnosis of DM for >25 years; however, specific information on glucose management or cardiac function during this period were not available.

VAP-1, argpyrimidine and Glo-I immunoreactivity in human ventricular tissues

In the majority of microvessels (100 to 250 μm) assayed from non-diabetic tissues, 66 out of 80, the endothelium was near continuous (intact) as determined from von Willerbrand factor immuno-reactivity (data not shown). This can also be seen as an intense green auto-fluorescence on the inside of microvessels (Figure 1A, upper two panels). In microvessels from Type 1 DM patients (>75 out of 90), the endothelial layer was disrupted (Figure 1A, third and fourth panels) and significantly less intense ($p=0.03$) than that in non-diabetic tissues. In the majority of microvessels (62 out of 77 vessels) from Type 2 DM patients, their endothelial layers were also near continuous. There was a trend towards less intense green auto-fluorescence in the endothelial layer of microvessels from Type 2 DM compared to non-diabetic, but the difference did not attain significance ($p=0.08$).

In coronary microvascular smooth muscle cells (cSMCs) from both T1DM and T2DM patients, VAP-1 immunoreactivity was 3- to 4-fold higher (Figures 1B, graph, $p<0.05$) and Glo-I immuno-reactivity was 2- to 2.5-fold lower than that in non-diabetic tissues (Figure 1A, upper graph, $p<0.05$). Argpyrimidine (one of the two adducts formed on arginine residues by MG) was also significantly higher ventricular tissues from T1DM and T2DM patients (Figure 1C).

VAP-1, argpyrimidine and Glo-I immunoreactivity in left ventricular tissues from DM rats

In ventricular tissues from 8 weeks DM rats VAP-1 was also increased in cSMCs while immuno-reactive Glo-I was reduced in cSMCs and myocytes (Figures 2A and 2B). Argpyrimidine immunoreactivity was also increased in ventricular tissues from DM rats (Figure 2C).

Having established dysregulation of the VAP-1 - MG – Glo-I axis in human and rat ventricular tissues, we proceeded to use a custom-engineered adeno-associated to prevent Glo-I downregulation in cSMCs and myocytes and determine if lowering MG production/flux would be sufficient to attenuate myocyte dysregulation and DC in our rat model of DM.

General characteristic of animals used in the study

The general characteristics of animals used in this study are summarized in Table 2. At the end of the 8 week experimental protocol, mean body weight of untreated DM rats was significantly lower than control rats. Heart weight to body weight ratio was also significantly higher ($p<0.05$) in DM rats compared with controls, and this increase was partially attenuated with systemic injection of AAV2/9-Endo-Glo-I and AAV2/9-CMV-Glo-I, but not AAV2/9-Endo-eGFP. There was a trend towards lower blood glucose levels in DM rats injected with AAV2/9-Endo-Glo-I, AAV2/9-CMV-Glo-I compared to DM but it did not attained statistical significance.

Well-being of animals

Nest building, which requires the complex interplay of attention, decision-making, motor function, and orofacial and forelimb movements, was used to assess overall well-being of animals at the end of the study. Eight week DM rats were less inclined to shred the cotton and create a

nest in the center of the cage overnight (Figure 2D, white arrow and graph below). The beddings of their cages were also "wetter" than controls, consistent with polydipsia/polyuria. Administering a single dose of AAV2/9-Endo-Glo-I one week after the onset of DM prevented impairment in overnight nest construction, assayed seven weeks later. DM rats treated with AAV2/9-Endo-Glo-I also drank ~25% less water overnight than untreated DM rats and exhibited a significant reduction in overnight polyuria, (see Figure 2D white arrow). Administering AAV2/9-CMV-Glo-I one week after the onset of DM, also preserved their ability to create a nest overnight in the middle of the cage, but it was not as robust like AAV2/9-Endo-Glo-I treated DM rats (Figure 2D). Although AAV2/9-CMV-Glo-I treatment reduced polydipsia/polyuria, it was not as robust as with AAV2/9-Endo-Glo-I treated DM rats. Injecting rats injected with a single dose of AAV2/9-Endo-eGFP one week after the onset of DM, did not preserve nest construction ability or reduced polydipsia/polyuria. Injecting control rats with AAV2/9-Endo-eGFP also not impair next building capabilities.

Left Ventricular Function

Peak flow velocity during early diastole (E), peak flow velocity during atrial contraction (A) and E-to-A ratios were decreased after 8 weeks of DM (representative examples shown in Figure 3A above, Table below shows mean data). M-Mode echocardiographic analyses (short axis) also showed significant reductions in left ventricular fractional shortening after 8 weeks of DM (Figure 3B). A single intravenous injection of AAV2/9-Endo-Glo-I one week after the onset of DM blunted the decrease in peak flow velocity during early diastole (E), peak flow velocity during atrial contraction (A) later in the disease. It also attenuated the loss in left ventricular fractional shortening.

A single intravenous injection of AAV2/9-CMV-Glo-I one week after the onset of DM also attenuated impairments in diastolic and systolic functions induced by diabetes. However, compared to DM rats injected with AAV2/9-Endo-Glo-I, AAV2/9-CMV-Glo-I was less effective ($p<0.05$) in some cardiac parameters including E:A ratio, heart rate and left ventricular diameter in diastole (see Tables below Figures 3A and 3B). As such, we focused on DM rats treated with AAV2/9-Endo-Glo-I for the rest of the study. A single intravenous injection of the non-specific AAV2/9-Endo-eGFP one week after the onset of DM did not attenuate loss of diastolic and systolic dysfunctions later in the disease.

Myocytes contractile kinetics

Myocytes from DM rats were less tolerant to Ca^{2+}reconstitution compared to myocytes from control animals and this resulted in about 20%-25% fewer viable ventricular myocytes compared to control. However, the mean lengths of DM myocytes from Con and DM rats were similar. When stimulated at a frequency of 0.5 Hz, velocities of shortening and relengthening of DM myocytes were significantly slower ($p < 0.05$) than those of control myocytes (Figure 4A, representation traces above and mean data in table below). Extent of shortening was also less in DM myocytes. Injecting rats with AAV2/9-Endo-Glo-I one week after the onset of DM blunted the changes in velocities of shortening and relengthening, and extent of myocyte shortening. Injecting rats with AAV2/9-Endo-eGFP one week after the onset of DM did not attenuate the reduction in myocyte function loss. Injecting control rats with AAV2/9-Endo-eGFP did not negatively impact myocyte contractile kinetics (Figure 4A, Table below**).**

Evoked intracellular Ca^{2+} transients

Improvements in contractile kinetics of myocytes from DM rats treated with AAV2/9-Endo-Glo-I prompted us to assess whether evoked Ca^{2+} release from the sarcoplasmic reticulum (SR) was improved. The efficiency of Ca^{2+} cycling out and into the SR is crucial for efficient and rhythmic contractions. In Figure 4B, upper panels show representative evoked Ca^{2+} transients pictograms from ventricular myocytes isolated from Con, DM and AAV2/9-treated Con and DM animals. The mean rate of evoked Ca^{2+} release from DM myocytes was significantly slower than that in control myocytes. In about 55% of DM myocytes assayed, evoked Ca^{2+} release was dyssynchronous (non-uniform) and spontaneous Ca^{2+} release occurred during diastole (Figure 4B pictograms, white arrows). Ca^{2+} transient decay time was also increased in DM myocytes (Figure 4B, Table below). Injecting rats with AAV2/9-Endo-Glo-I one week after the onset of DM blunted the reductions in evoked Ca^{2+} release from and reuptake into the SR seven weeks later. It also blunted dyssynchronous/non-uniform Ca^{2+} release from the SR and spontaneous Ca^{2+} release occurred during diastole. Injecting rats with AAV2/9-Endo-eGFP one week after the onset of DM did not blunt the reductions in evoked Ca^{2+} release from and reuptake into the SR. Injecting control rats with AAV2/9-Endo-eGFP did not affect evoked Ca^{2+} release from and reuptake into the SR. Summary data are shown in Table below Figure 4B.

Microvessel perfusion and permeability

In left ventricular tissues from control rats, the green fluorescence of injected BSA-FITC was visible throughout the entire microvascular networks (Figure 5A, upper panel), indicating that the tissues were efficiently being perfused with blood/nutrients. The green fluorescence of BSA-FITC was also confined to vascular networks. The first noticeable difference in left ventricular tissues from DM rats was that the green fluorescence of BSA-FITC was diffusing out of the

microvessels, consistent with "leakage" or increased transcytosis (Figure 5A, middle panel). The density of microvessels perfused with BSA-FITC (i.e., with green fluorescence) was also significantly reduced ($p<0.05$, Figure 5A middle panel and graphs), suggesting under perfusion/reduced perfusion of the ventricular microvasculature in DM rats. Administration of a single intravenous injection of AAV2/9-Endo-Glo-I to rats one week after the onset of DM blunted loss of microvessel perfusion prevented the leakiness of the microvessels (Figure 5A lower panel, and graphs). This increase in microvasculature perfusion was not seen in DM rats injected with AAV2/9-Endo-eGFP (Figure 5A). Injecting control rats with AAV2/9-Endo-eGFP did not alter microvasculature perfusion or permeability.

Expression/activities of VAP-1 and Glo-I

In ventricular tissues from 8 week DM rats, Glo-I levels was reduced 3-fold and VAP-1 was increased 3.2-fold (Figure 5B). Treating rats with AAV2/-Endo-Glo-I one week after the onset of DM, blunted VAP-1 upregulation and preserved Glo-I expression later in the disease. Treating rats with AAV2/-Endo-eGFP one week after the onset of DM, did not prevent diabetes-induced alterations in VAP-1 and Glo-I expression (data not shown).

Glo-I activity, determined by the formation of S-D-lactoylglutathione formation, was also ~50 % lower in ventricular tissues from DM rats (Table 2). Injecting rats with AAV2/9-Endo-Glo-I one week after the onset of DM, minimally lowered serum MG. However, it significantly reduced MG level in ventricular tissue ($p<0.05$, Table 2) to near that in control animals and the blunted loss of Glo-I activity. Table 2, lower row also shows increased MG levels in hearts of DM rats that was blunted following a single injection with AAV2/-Endo-Glo-I one week after the onset of DM, prevented the increase in MG.

Expression and activities of SR Ca^{2+} cycling proteins

Improvements in contractile kinetics and evoked Ca^{2+} transients following AAV2/9-Endo-Glo-I treatment also prompted us to measure the activities of the major SR Ca^{2+} release and uptake proteins, RyR2 and SERCA2a/PLN in myocytes.

(a) SERCA2a: Steady state level of SERCA2a and its inhibitor protein phospholamban (PLN) in 8 week DM rats were not significantly different from controls (Figure 5C). There was also no significant change in the amount of phospho-PLN (Ser16, phospho-Ser 17, Figure 5C, autogradiograms). However, the abilities of SERCA2a from DM rats to transport Ca^{2+} ($E_1 \rightarrow E_2$) and hydrolyze ATP were significantly lower than that of control animals (Figures 5C and 6A, $p<0.05$). Administering a single intravenous dose of AAV2/9-Endo-Glo-I one week after the onset of DM also did not alter expression of SERCA2a, monomeric PLN, and pentameric PLN but it attenuated the loss in SERCA2a's ability to transport Ca^{2+} ($E_1 \rightarrow E_2$) and hydrolyze ATP. Injecting rats with AAV2/9-Endo-eGFP one week after the onset of DM did not attenuate SERCA2a activity loss. Injecting control rats with AAV2/9-Endo-eGFP did not impair the ability to transport Ca^{2+} and hydrolyze ATP (Figures 5C and 6A).

(b) RyR2: After 8 weeks of DM, steady-state levels of RyR2 protein in DM hearts was not significantly different from control (Figure 6B). However, the Ca^{2+}-responsiveness (function) of RyR2 as assessed from its ability to bind the specific ligand [^{3}H]ryanodine was altered (Figure 6B). At suboptimal Ca^{2+} (≤ 40 μM), RyR2 from DM animals bound more [^{3}H]ryanodine after 2 hrs, consistent with enhanced activity/opening at low [Ca^{2+}]. Interestingly, at optimal Ca^{2+} (200-300 μM), RyR2 from DM animals bound significantly less [^{3}H]ryanodine than RyR2 from control animals, indicative of reducing activity/opening. Injecting rats with AAV2/9-Endo-Glo-I one

week after the onset of DM restored Ca^{2+} activation of RyR2. Injecting rats with AAV2/9-Endo-eGFP one week after the onset of DM did not restore Ca^{2+} sensitivity. Injecting control rats with AAV2/9-Endo-eGFP did not affect the Ca^{2+}-sensitivity of RyR2 as measured using [^{3}H]ryanodine binding assays, (Figure 6B).

(c) MG adduct on RyR2 and SERCA2a: Previously we showed that impairments in activities of RyR2 and SERCA2a in hearts of chronic DM rats resulted in part from increased post-translational modifications by MG (29, 39). Since treatment of DM rats with AAV2/9-Endo-Glo-I attenuated the increase in ventricular MG, we assessed formation of MG-adduct on RyR2 and SERCA2a were also attenuated. Figures 6C and 6D show ~3-fold higher amounts of the argpyrimidine (an MG-derived adduct) on SERCA2a and RyR2 from DM animals compared with controls. Injecting rats with AAV2/9-Endo-Glo-I one week after the onset of DM, attenuated formation of argpyrimidine adduct on SERCA2a and RyR2. Injecting DM rats with AAV2/9-Endo-eGFP did not attenuate the amount of argpyrimidine on SERCA2a and RyR2. Injecting control rats with AAV2/9-Endo-eGFP one week after the onset of DM did not affect the formation of argpyrimidine adducts on SERCA2a and RyR2 (data not shown).

Glutathione (GSH) and enzymes that regulate GSH level

Since reduced glutathione (GSH) is a co-factor for Glo-I, GSH and GSSH levels, and the activities of the enzymes that regulate GSH levels were measured in heart tissues. In this study, GSH:GSSG ratio was significantly lower ($p<0.05$) in ventricular tissues from DM rats compared with controls (1.55 ± 0.05, $n = 5$ hearts compared with 2.4 ± 0.2, $n = 5$ hearts). Injecting rats with AAV2/9-Endo-Glo-I one week after the onset of T1D, increased GSH/GSSG to near control

levels (2.3 ± 0.3, n = 6 hearts). Administering AAV2/9-GFP to DM rats did not potentiate GSH/GSSG ratio (1.6 ± 0.3, n = 5 hearts).

In hearts of DM rats (γ-glutamylcysteine ligase, the rate-limiting enzyme in the synthesis of GSH) and glutathione reductase (the enzyme that reduces oxidized glutathione) were decreased by 35% and 32%, respectively compared with control (205.2 ± 1.2 mU/mg protein compared to 470.5 ± 18.1 mU/mg protein and 32.5 ± 8 mU/mg protein compared to 48.1 ± 7.4 mU/mg protein). Administering AAV2/9-Endo-Glo-I to DM rats restored the activities of γ-glutamylcysteine ligase and glutathione reductase to near control levels (460 ± 20 mU/mg protein and 45.5 ± 10.1 mU/mg protein, respectively). Administering AAV2/9-Endo-eGFP to DM rats did not significantly increase the activities of γ-glutamylcysteine ligase and glutathione reductase (210.5 ± 21.2 mU/mg protein and 30.5 ± 11.2 mU/mg).

ROS and inflammation in ventricular tissues

Earlier we showed that MG is a potent inducer of mitochondria ROS in cardiac myocytes (29). After 8 weeks of DM, basal mitochondria ROS was increased ~3.5 fold in ventricular myocytes (Figure 7A). Treating rats with AAV2/9-Endo-Glo-I one week after induction of DM, attenuated the increase in mitochondria ROS in ventricular myocytes. Treating DM rats with AAV2/9-Endo-Glo-I one week after induction of DM, In this study, mRNA level for the inflammatory marker TNF-α was 3.2-fold higher in left ventricular tissues of DM rats compared to control ($p<0.05$, Figure 7B). Figure 7C also shows a 3.8-fold increase in the amount of p-p65 of NF-κB in 8 week DM rats compared to control ($p<0.05$). Injecting DM rats with AAV2/9-Endo-Glo-I significantly blunted the amount of TNF-α mRNA and p65 phosphorylation. Injecting DM rats

with AAV2/9-Endo-eGFP minimally attenuated the increase in p-p65 and TNF-α mRNA (data not shown).

Gregg et al., (48) recently reported that although the incidence of micro- and macro-vascular diseases has declined in patients with DM over the past two decades, they are still 5-7 times higher than in non-DM individuals. These epidemiologic data suggest that additive therapies may be needed to complement glucose and lipid lowering agents if end-organ complications are to be reduced, especially in outpatient settings. One potential strategy could be to lower the supra-physiological MG flux thought to be eliciting many of the pathobiologies seen in DC.

Earlier, we showed that daily administration of scavengers of reactive carbonyl species (aminoguanidine and pyridoxamine) to DM rats attenuated DC development (29, 36). Vulesevic et al., (49) also showed that increasing expression of the MG degrading enzyme Glo-I in bone marrow-derived macrophages and endothelial cells, protected against DC. Very recently, Xue et al., (50) showed that administering a Glo-I inducer formulation (trans-resveratrol and hesperetin) to overweight and obese individuals for eight weeks attenuated fasting and post prandial glucose and inflammation, and improved arterial dilation. Since low MG regulates a diverse array of cellular and physiologic processes, and since DM requires lifelong treatment, we extended the work to identify "the source" of heart failure-causing MG. Glycolysis leak is unlikely since the primary source since the diabetic heart obtains 90-95% of the acetyl CoA needed for ATP production from β-oxidation of fatty acids (30).

A key finding of the present study is that MG production/flux is increased in the heart during DM and this increase is arising from upregulation of VAP-1 in cSMCs (4-5 fold), and from reductions in Glo-I (3-4 fold) in SMCs and mycoytes. These changes were seen in both Type 1 and Type 2 DM, likely reflecting increases in oxidative stress and inflammation (46, 47). They also complement data from other studies (35, 51, 52). In this study we also showed that for the first time that preventing Glo-I downregulation in cSMCs and ventricular myocytes using a

custom-designed adeno-associated virus, attenuated DC development in a rat model of DM, establishing a direct relationship between increase in ventricular MG and heart failure. Attenuation in myocyte and cardiac function losses were also independent of blood glucose levels.

In this study we found that preserving Glo-I levels it attenuated microvascular leakage, inflammation, loss of microvessel perfusion (hypoxia) in left ventricular tissues. It also blunted impairments in blood flow velocity during early diastole (E) and blood flow velocity during atrial contraction. Dysregulation of endothelial cells (ECs) plays an important role in the pathogenesis of DC by altering vasodilator tone and permeability of microvessels (51). When ECs lose their ability to vasodilate pressure builds up within the vasculature, increasing the likelihood of substances from the blood leaking into the surrounding tissues. This was seen as green fluorescence (of BSA-FITC) emanating from small diameter arterioles (20 μm - 50 μm) and capillaries in left ventricular tissue of rats. Why some but not all vessels become "leaky" is not well defined. One explanation could be heterogeneity in VAP-1 expression and therefore the amount of MG produced by SMCs; MG impairs the function of ECs in a concentration-dependent manner (35). Although capillaries do not contain SMCs, the function of their ECs could be negatively impacted by MG produced by cSMCs in upstream arterioles and met-arterioles. In this study we also found a significant reduction in the density of microvessels perfused, suggesting impairment in nutrient supply to the myocytes. This reduction in the density of microvessel perfusion could be the results of increased leakage of microvessels.

Preventing Glo-I downregulation also preserved myocyte function. It blunted loss of ventricular contraction/relaxation, impairments in evoked contraction/relaxation kinetics, and dysregulation of Ca^{2+} cycling into and out of the SR by prevented dysregulation of RyR2 and

SERCA2 and reducing oxidative stress. In this, MG production/flux in ventricular tissues from DM rats increased 8-fold (1585 nmol/200 mg tissue compared to 198 nmol/200 mg tissue for control). At a first glace these results seems higher than the expected a 3-4 fold increase in MG based on the 3-4 fold increase in VAP-1 protein. However, it should be pointed out that although VAP-1 and triose phosphate isomerase have similar affinities for their substrates (K_m of 92 μM vs ~120 μM), the V_{max} for VAP-1 is >2-orders of magnitude faster than that of triose phosphate isomerase (54-55). Also, since reduced glutathione levels in the extracellular environment are low, significantly more of the MG produced by VAP-1 in the extracellular will be in the unbound state compared to MG inside cells (56). Earlier we showed that exposing rat ventricular myocytes to 20-25 μM MG perturb SR Ca^{2+} and increase mitochondria ROS production (29).

To date, the specific mechanisms by which increasing Glo-I in cSMCs of DM rats down-regulated VAP-1 expression remains undefined. What we know is that the VAP-1 gene (*AOC3*) is transcriptionally regulated by NF-κB (57) and that MG activates NF-κB (58). Reducing MG in cSMCs by increasing Glo-I prevents NF-κB activation (p-P65) which in turn blunts VAP-1 upregulation. Preventing Glo-I downregulation also blunted TNF-α (inflammation) increase in hearts of DM rats. Although we did not specifically assess macrophage and neutrophil infiltration the primary sources of TNF-α production, we did assess "leakiness/permeability" of microvessels, which promotes macrophage and neutrophil infiltration in DM rats (35, 49, 59).

AAV serotypes exhibit selective tropism for certain cell types. In the heart, AAV2/9 shows preferential tropism for SMCs and myocytes (35, 60). Endothelin-1 is also synthesized by macrophages. By cloning the endothelin-1 promoter in front of Glo-I cDNA, we were able to selectively target expression of Glo-I protein in SMCs and myocytes in the heart. Using this custom-designed virus, we found that preventing Glo-loss in ventricular tissues (SMCs and

myocytes) of DM rats was sufficient to blunt DC development. What if any would be the consequences of increasing Glo-I in SMCs and myocytes of DM to levels significantly greater than that in non-diabetics remain to be determined. In the vasculature, endothelin-1 is synthesized by smooth muscle cells and endothelial cells (61). By cloning the endothelin-1 promoter in front of Glo-I cDNA, we were able to preferentially target expression of Glo-I protein in the heart to SMCs and myocytes. Our custom-designed virus restored Glo-I protein in ventricular tissues of Type 1 DM rats to levels similar to that in Con rats. What if any would be the functional consequences of increasing Glo-I in hearts of DM to levels significantly greater than that in non-diabetics remains to be determined. Also, it is not clear whether increasing Glo-I in cSMCs only would be sufficient to attenuate heart failure.

In the present study we also found that single injection of AAV2/9-Endo-Glo-I one week after the onset DM significantly reduced polydipsia and overnight polyuria seven weeks later. It also prevented impairment in nest construction, which requires the complex interplay of attention, decision-making, motor function, and orofacial and forelimb movements. Although the mechanism(s) responsible for these effects are not clear, we expect systemic injection of AAV2/9-Endo-Glo-I will infect SMCs (and possible other cells) throughout the vasculature. These are exciting observations that could have implications in the management of other end-organ deficits in DM.

In summary, the present study shows that MG production/flux is increased in the heart during DM, and this increase arises from upregulation of VAP-1 in cSMCs (4-5 fold), and from reductions in Glo-I (3-4 fold) in SMCs and mycoytes. This increase in MG production/flux leads to increased vascular permeability, a reduction in density of micro-vessels perfused, inflammation, and oxidative stress. It also results in post-translational modification and

dysregulation of RyR2 and SERCA2 (and possibly other proteins), perturbation in myocyte intracellular Ca^{2+} cycling and impairment in myocyte contraction/relaxation (Figure 7D). Preserving Glo-I protein in cSMCs and myocytes blunted DC development.

ACKNOWLEDGEMENTS

This work was supported in part by the Gene Therapy Research Program, GTRP 1053 and by the Nebraska Redox Biology Center, Lincoln NE (P30GM103335). Dr. Keshore R. Bidasee is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

AUTHORS CONTRIBUTION

K.R.B. came up with the hypothesis and designed the study in conjunction with J.S and G.J.R. Autopsied human samples came from tissue banks at UNMC. F.M.A., R.F., and K.R.B performed experiments and analyzed data. K.R.B wrote the manuscript. G.J.R. and J.S edit the manuscript. The authors thank Dr. Chun Hong Shao for analysis of some of the initial samples. We also thank Janice A. Taylor and James R. Talaska (Confocal Laser Scanning Microscope Core Facility, University of Nebraska Medical Center)

No potential conflicts of interest exist.

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FIGURE LEGENDS

Table1: Overview of patient samples used in the study. Values shown are mean ± S.E.M. for n = 15-19 samples where indicated. * denotes significant difference from non-diabetic ($p<0.05$).

Table 2: General characteristics of animals used for the study. Values shown are mean ± S.E.M. for n = ≥14 animals per group. * denotes significant difference from Con ($p<0.05$), # denotes significant difference from DM ($p<0.05$).

Figure 1: Steady-state levels of VAP-1, Glo-I, and argpyrimidine (MG adduct formed on arginine residues or proteins) in coronary microvessels from left ventricular tissues from non-diabetic and diabetic patients. **Panel A** shows representative immuno-histochemical staining for alpha smooth muscle actin (SM22-α, red) and VAP-1 (green) in smooth muscle cells from autopsied left ventricular tissues from two non-diabetic (upper two), two Type 1 DM (middle two) and two Type 2 DM (lower two) patients**.** Graph on right shows mean ± S.E.M. for n ≥50 vessels obtained from N≥3 patients per group. **Panel B** shows representative immuno-histochemical staining for alpha smooth muscle actin (SM22-α, red) and Glo-I (green) in smooth muscle cells from autopsied left ventricular tissues from two non-diabetic (upper two), two Type 1 DM (middle two) and two Type 2 DM (lower two) patients. Graph on right show mean ±

S.E.M. for n ≥50 vessels obtained from N≥3 patients per group. **Panel C** shows representative immuno-histochemical staining for alpha smooth muscle actin (SM22-α, green) and argpyrimidine (red) in smooth muscle cells and myocytes from autopsied left ventricular tissues from two non-diabetic (upper two), two Type 1 DM (middle two) and two Type 2 DM (lower two) patients**.** Graph on right shows mean ± S.E.M. for n ≥50 vessels obtained from N≥3 patients per group. White bar at bottom of images = 50 μm. White arrows are included to emphasize the presence/lack of over overlapping staining. * denotes significant difference from Con ($p<0.05$).

Figure 2: Steady-state levels of VAP-1, Glo-I, and argpyrimidine (MG surrogate) in coronary microvessels from left ventricular tissues from non-diabetic and diabetic rats. **Panel A** shows representative immuno-histochemical staining for alpha smooth muscle actin (SM22-α, red) and VAP-1 (green) in smooth muscle cells from left ventricular tissues from two non-diabetic (upper two) and two STZ-Type 1 DM rats**.** Graph on right show mean ± S.E.M. for n ≥40 vessels obtained from N≥5 animals per group. **Panel B** shows representative immuno-histochemical staining for alpha smooth muscle actin (SM22-α, red) and Glo-I (green) in smooth muscle cells from left ventricular tissues from two non-diabetic (upper two) and two STZ-Type 1 DM rats**.** Graph on right show mean ± S.E.M. for n ≥40 vessels obtained from N≥5 animals per group. **Panel C** shows representative immuno-histochemical staining for alpha smooth muscle actin (SM22-α, green) and argpyrimidine (red) in left ventricular smooth muscle cells and myocytes from tissues from two non-diabetic (upper two) and two STZ-Type 1 DM rats**.** Graph on right show mean ± S.E.M. for n ≥40 vessels obtained from N≥5 animals per group. White bar at bottom of images = 50 μm. White arrows are included to emphasize the presence/lack of over

overlapping staining. **Panels D** shows overnight nest construction of animals used in the study. Graph below shows mean ± S.E.M. for n = 5-6 animals per group. For all panels, * denotes significant difference from Con ($p<0.05$), and # denotes significant difference from DM ($p<0.05$).

Figure 3: *In vivo* cardiac function of hearts from control, DM and DM-treated rats. Panel A upper shows representative pulse-wave echocardiograms of hearts from control, DM, AAV2/9-Endo-Glo-I-treated DM and AAV2/9-CMV-Glo-I-treated DM rats. For this, rats were anesthetized with 3% isoflurane and after securing the rats with tape in the supine position on a heated (37°C) pad, maximal early (E) and late (A) diastolic transmitral flow velocities were measured in the short axis orientation using a color flow–guided, pulsed-wave Doppler probe (716, 17.6 MHz). Mean ± S.E.M data for peak flow velocity during early diastole (E), peak flow velocity during atrial contraction (A) and E: A ratios for n = 18-20 animals are listed in the table below. **Panel B upper** shows representative M-mode echocardiograms from control, DM, AAV2/9-Endo-Glo-I-treated DM and AAV2/9-CMV-Glo-I-treated DM rats. For this, rats were anesthetized with 3% isoflurane and a 4MHz linear array pediatric probe was then placed in the parasternal, short axis orientation while maintaining normothermia with a heating pad. Three loops of M-mode data were captured for each animal, and the data was averaged from at least 5 beat cycles/loop. Mean ± S.E.M hemodynamic data for n = 18-20 animals are listed in the table below.

Figure 4: Function of left ventricular myocytes from control, DM and DM-treated rats. Panel A shows representative contractile profile of ventricular myocyte isolated from control, AAV2/9-Endo-eGFP-treated Con, DM, AAV2/9-Endo-eGFP-treated DM and AAV2/9-Endo-Glo-I-treated DM rats. For this, myocytes in DMEM medium were placed in a Warner chamber mounted on the stage of an inverted microscope (Zeiss, X-40) at room temperature (22°C–24°C). Cells were then field stimulated (10 V) for 10 ms at 0.5 Hz, using a pair of platinum wires placed on opposite sides of the chamber. Extent of myocyte shortening and rates of shortening and relengthening were determined using IonWizard Version 5.0. Mean ± S.E.M for n >120 cells from N= 5 animals are listed in the table below. **Panel B** shows representative line scan images of Ca^{2+} transients from control, AAV2/9-Endo-eGFP-treated Con, DM, AAV2/9-Endo-eGFP-treated DM and AAV2/9-Endo-Glo-I-treated DM rats preloaded with Fluo-3AM and field stimulated at 0.5 Hz. White arrows in indicate regions of dyssynchronous (non-uniform) Ca^{2+} from the SR and diastolic Ca^{2+} release. Mean ± S.E.M for n >80 cells from N= 5 animals are listed in the table below. For all panels, * denotes significant difference from Con ($p<0.05$), and # denotes significant difference from DM ($p<0.05$).

Figure 5: Panel A left shows representative images of BSA-FITC in left ventricular tissues from Con, DM, and AAV2/9-Endo-Glo-I-treated DM rats. For this, rats were injected with BSA-FITC (40 mg/kg) 10 min prior to sacrifice. After this, animals were sacrificed, and hearts were removed and immersed in 4% paraformaldehyde for 24 hrs followed by 30% sucrose. Twenty micrometer longitudinal sections, were then placed on pre-cleaned glass slides, mounting medium containing DAPI was added and images were then taken at 100X. Graphs on right side show relative density of microvessel (>20 μm) perfused with BSA-FITC per 100X frame and the

number of microvessels from which BSA-FITC was seen emanating. Values are mean ± S.E.M from n >20 sections from N= 5 rats. White bar at bottom of each image = 50 μm. **Panel B** shows representative autoradiograms for VAP-1 and Glo-I in hearts from Con, DM, and AAV2/9-Endo-Glo-I-treated DM rats, normalized to β-actin. Data shown in graph below are mean ± S.E.M from N= 5 rats done in duplicate. **Panel C upper** shows representative autoradiograms for SERCA2a, monomeric and pentameric PLN and β-actin in hearts from control, AAV2/9-Endo-eGFP-treated Con, DM, AAV2/9-Endo-eGFP-treated DM and AAV2/9-Endo-Glo-I-treated DM rats. Data shown in graph below are mean ± S.E.M (N= 5 rats done in triplicate) for ATP-stimulated Ca^{2+} uptake (SERCA2a function) in vesicular preparations from control, AAV2/9-Endo-eGFP-treated Con, DM, AAV2/9-Endo-eGFP-treated DM and AAV2/9-Endo-Glo-I-treated DM rats.

Figure 6: Panel A shows mean ± S.E.M (N= 5 rats done in triplicate) for ATP hydrolysis (SERCA2a function) in vesicular preparations from control, AAV2/9-Endo-eGFP-treated Con, DM, AAV2/9-Endo-eGFP-treated DM and AAV2/9-Endo-Glo-I-treated DM rats. Data shown in graph below are mean ± S.E.M from N= 5 rats done in duplicate. **Panel B** shows Ca^{2+}-dependent binding [^{3}H]ryanodine to RyR2 from control, AAV2/9-Endo-eGFP-treated Con, DM, AAV2/9-Endo-eGFP-treated DM and AAV2/9-Endo-Glo-I-treated DM rat hearts. Significance notations were left out to aid in data clarity. For all panels, * denotes significant difference from Con ($p<0.05$), and # denotes significant difference from DM ($p<0.05$). **Panel C upper** shows representative autoradiograms for argpyrimidine on SERCA2 from in SR membrane preparations from control, DM, and AAV2/9-Endo-Glo-I-treated DM rat hearts. Data shown in graph below are mean ± S.E.M for N= 5 separate preparations assayed in duplicate. **Panel D upper** shows

representative autoradiograms for argpyrimidine on RyR2 from in SR membrane preparations from control, DM, and AAV2/9-Endo-Glo-I-treated DM rat hearts. Data shown in graph below are mean ± S.E.M for N= 5 separate preparations assayed in duplicate.

Figure 7: Panel A left shows representative Mitotracker Green (mitochondria) and MitoSox red (ROS) staining in left ventricular myocytes isolated from control, DM, and AAV2/9-Endo-Glo-I-treated DM rat hearts. Data in graph on right are mean ± S.E.M for MitoSox staining obtained from >40 myocytes obtained N= 5 animals. **Panel B** shows relative amounts of TNF-α mRNA in left ventricular tissues from control, DM, and AAV2/9-Endo-Glo-I-treated DM rat hearts. Values shown in graph are mean ± S.E.M from N = 5-6 rats per group, normalized to actin. **Panel C** shows representative autoradiograms for phosphorylated NF-κB (p65) in left ventricular lysates from control, DM, and AAV2/9-Endo-Glo-I-treated DM rat hearts. Values shown in graph below are mean ± S.E.M from N = 5-6 rats per group. **Panel D shows** a schematic model summarizing data obtained from this study and a proposed mechanism by which increased production of MG by SMCs lead to the development of DC.

Table 1: Overview of patient samples used in the study

Non-DIABETIC						
Number of samples	**Mean age (yrs)**	**Males**	**Female**	**Duration of DM**	**HbA1c (%)**	**Time to tissue collection (hr)**
15	**63.3 ± 3.9**	**10**	**5**		**4.5 ± 1.4**	**10.9 ± 2.4**
DIABETIC						
Number of samples	**Mean age (yrs)**	**Males**	**Female**	**Duration of DM**	**HbA1c (%)**	**Time to post mortem (hr)**
19	**68.8 ± 2.1**	**11 (three Type 1 and eight Type 2)**	**8 (one Type 1, seven Type 2)**	**26.5 ± 5.9**	**7.25 ± 0.5**	**9.9 ± 2.9**

Table 2: General characteristics of animals used in the study

PARAMETER	Control n = 14	Control treated withAAV2/9-Endo-eGFP n = 14	Diabetic n = 20	Diabetic treated with AAV2/9-Endo-Glo-I n = 20	Diabetic treated with AAV2/9-Endo-Glo-I n = 20	Diabetic treated with AAV2/9-CMV-Glo-I n = 18
Body mass (g)	415.2 ± 10.2	401.3 ± 11.4	275.4 ± 15.2*	290.4 ± 12.3*	320.3 ± 10.3#	315.2 ± 15.2#
Heart weight	1.2 ± 0.2	1.2 ± 0.2	1.0 ± 0.3	1.0 ± 0.3	1.1 ± 0.2	1.1 ± 0.2
Heart to body weight ratio (mg/g)	3.0 ± 0.2	3.0 ± 0.2	3.6 ± 0.3	3.5 ± 0.3	3.4 ± 0.3	3.4 ± 0.3
Blood glucose (mmol)	5.5 ± 1.6	6.3 ± 1.7	25.6 ± 4.5*	24.1 ± 3.5	22.9 ± 3.2	22.1 ± 4.2
% Glycated hemoglobin	4.2 ± 0.3	4.3 ± 0.4	7.6 ± 0.2*	7.5 ± 0.3*	7.2 ± 0.5	7.3 ± 0.6#
Serum insulin (ng/mL)	0.9 ± 0.2	1.0 ± 0.1	0.3 ± 0.1*	0.3 ± 0.1*	0.6 ± 0.1#	0.4 ± 0.2
Serum TBARS[1] (nmol/mL)	2.4 ± 0.3	2.5 ± 0.6	12.0 ± 2.1*	11.5 ± 1.9*	7.5 ± 1.0#	9.8 ± 1.2
SSAO activity[2] (units/mL/min)	0.3 ± 0.02	0.3 ± 0.03	0.7 ± 0.1*	0.7 ± 0.1*	0.6 ± 0.0#	0.6 ± 0.0#
Serum MG (µM)	0.3 ± 0.1	0.3 ± 0.1	1.2 ± 0.2*	1.4 ± 0.2*	1.2 ± 0.3	1.4 ± 0.2#
Ventricular MG (nmol/200 mg)	197.8 ± 34.2	203.7 ± 46.5	1588.5 ± 174.7*	1790.7 ± 205.8*	184.4 ± 30.2#	475.5 ± 110.7#
Ventricular Glyoxal (nmol/200 mg)	252.8 ± 32.2	330.6 ± 90.5	3110.5 ± 184.7*	3326 5 ± 205.8*	356.4 ± 30.2#	654.5 ± 95.7#
Glo-I activity (µmol/min/100 mg ventricular tissue)	10.2 ± 1.0	11.4 ± 1.0	5.1 ± 0.8*	6.3 ± 0.6*	13.2 ± 1.2#	12.1 ± 1.6#
VAP-1 activity (units/min/100 mg ventricular tissue)	0.2 ± 0.0	0.2 ± 0.0	1.4 ± 0.1*	1.4 ± 0.1*	0.4 ± 0.0#	0.5 ± 0.1#

*** = significantly different from control ($p<0.05$), # = significantly different from diabetic ($p<0.05$)**

1 - TBARS, Thiobarbituric acids reactive substances; 2 - SSAO, semicarbazide-sensitive amine oxidase

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8